

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/82, A01H 5/00, 5/10		A2	(11) International Publication Number: WO 00/37663 (43) International Publication Date: 29 June 2000 (29.06.00)
(21) International Application Number: PCT/US99/30972 (22) International Filing Date: 23 December 1999 (23.12.99)		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(30) Priority Data: 60/113,717 23 December 1998 (23.12.98) US 60/145,373 23 July 1999 (23.07.99) US		Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(71) Applicant (for all designated States except US): THE SAMUEL ROBERTS NOBLE FOUNDATION, INC. [US/US]; 2510 Sam Noble Parkway, Ardmore, OK 73401 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): HARRISON, Maria, J. [GB/US]; HC60, Box 26A1, Ardmore, OK 73401 (US). TRIEU, Anthony, T. [US/US]; 9330 Zionsville Road, Indianapolis, IN 46268 (US).			
(74) Agents: HANSEN, Eugenia, S. et al.; Sidley & Austin, Suite 3400, 717 North Harwood, Dallas, TX 75201 (US).			
(54) Title: PLANT TRANSFORMATION PROCESS			
(57) Abstract			
<p>A method of <i>Agrobacterium</i>-mediated genetic transformation using seedlings has been found which is applicable to dicots and monocots capable of being transformed by <i>Agrobacterium</i>. The transformation method utilizes vacuum-infiltration to introduce the <i>Agrobacterium</i> T-DNA carrying a gene of interest into the seedlings. Upon maturity, seeds collected from the infiltrated seedlings are germinated, and progeny carrying the transgene are selected. This transformation method produces progeny exhibiting stable inheritance of the transgene without the need for regeneration methods such as somatic embryogenesis or organogenesis.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

PLANT TRANSFORMATION PROCESS**BACKGROUND**

Genetic transformation of higher plants promises to have a major impact on crop improvement, as well as many other areas of biotechnology. Genetic transformation can be used to produce transgenic plants carrying new genetic material stably integrated into the genome and to engineer 'designer' crops with specific traits. Various methods of genetic transformation have been developed and applied to a growing number of plant species. However, the ease and success rate of genetic transformation methods varies widely among plant species [Muller, et al. 1987. "High meiotic stability of a foreign gene introduced into tobacco by *Agrobacterium*-mediated transformation," *Mol Gen Genet* 207:171-175; Gasser, C.S. and Fraley, R.T. 1989. "Genetically engineering plants for crop improvement," *Science* 244:1293-1299; Umbeck, et al. 1989. "Inheritance and expression of genes for kanamycin and chloramphenicol resistance in transgenic cotton plants," *Crop Science* 29:196-201; Gordon-Kamm, et al. 1990. "Transformation of maize cells and regeneration of fertile transgenic plants," *Plant Cell* 2:603-618; Chabaud, et al. 1996. "Transformation of barrel medic (*Medicago truncatula* Gaertn.) by *Agrobacterium tumefaciens* and regeneration via somatic embryogenesis of transgenic plants with *MtENOD12* nodulin promoter fused to the *gus* reporter gene," *Plant Cell Rep* 15:305-310; Kar, et al. 1996. "Efficient transgenic plant regeneration through *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.)," *Plant Cell Rep* 16:32-37; Kim, J.W. and Minamikawa, T. 1996. "Transformation and regeneration of French bean plants by the particle bombardment process," *Plant Sci* 117:131-138; Trieu, A.T. and Harrison, M.J. 1996. "Rapid transformation of *Medicago truncatula*: regeneration via shoot organogenesis," *Plant Cell Rep* 16:6-11; Bean, et al. 1997. "A simple system for pea transformation," *Plant Cell Rep* 16:513-519; Cheng, et al. 1997. "Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*," *Plant Physiol* 115:971-980 ; Cheng, et al. 1997. "Expression and inheritance of foreign genes in transgenic peanut plants generated by *Agrobacterium*-mediated transformation," *Plant*

Cell Rep 16:541-544; and Tingay, et al. 1997. "Agrobacterium tumefaciens-mediated barley transformation," *Plant J* 11:1369-1376.]

The most common and widely used method of transformation of dicotyledonous plants utilizes a bacterium, *Agrobacterium tumefaciens*, to effect gene transfer.

5 *Agrobacterium tumefaciens* is a gram-negative, soil dwelling plant pathogen that infects its plant host and subsequently delivers and integrates part of its genetic material into the plant genome. The transferred portion of DNA is termed the T-DNA fragment, and additional genetic material can be added to the T-DNA. The additional genetic material will then be integrated into the genome along with the T-DNA. In this way, 10 *Agrobacterium* can be used to facilitate the transfer of new genes into the plant genome (Fraley, et al. 1983. "Expression of bacterial genes in plant cells," *Proc Natl Acad Sci USA* 80:4803-4807).

15 While transformation with *Agrobacterium* has worked well for a number of model species such as tobacco and petunia, the approach is subject to a number of limitations. Some plant species, including many monocotyledonous plant species, are not readily susceptible to infection by *Agrobacterium* (Potrykus, I. 1990. "Gene transfer to cereals: an assessment," *Bio/Technology* 8:535-542). In these cases, alternative approaches have been used, including particle bombardment and direct gene transfer into protoplasts via electroporation, microinjection, or polyethylene glycol mediated uptake [Klein, et al. 1987. "High velocity microprojectiles for delivering nucleic acids into living cells," *Nature* 327:70-73; McCabe, et al. 1988. "Stable transformation of soy bean (*Glycine max*) by particle acceleration," *Bio/Technology* 6:923-926; Bommineni, et al. 1994. "Expression of GUS in somatic embryo cultures of black spruce after 20 microparticle bombardment," *J Exp Bot* 45:491-495; Christou, P. 1995. "Strategies for variety-independent genetic transformation of important cereals, legumes and 25 woody species utilizing particle bombardment," *Euphytica* 85:13-27; Kim and Minamikawa. 1996. *Plant Sci* 117:131-138; Klein, et al. 1998. "Stable genetic transformation in intact *Nicotiana* cells by the particle bombardment process," *Proc Natl Acad Sci USA* 85:8502-8505].

Regardless of the method of delivery of the new genetic material, it is necessary to regenerate whole fertile plants from the transformed cells. The production of stably transformed transgenic plants involves two processes: transformation of plant cells and then regeneration of those transformed cells to whole plants. In most cases, a plant tissue explant is incubated with *Agrobacterium* carrying a T-DNA containing a selectable marker gene and a 'gene of interest'. A proportion of the cells in the explant will become transformed, and whole plants are then regenerated from these cells via somatic embryogenesis or direct organogenesis. Transformants are selected by inclusion of the appropriate selective conditions in the regeneration media. The choice of tissue explant depends on the plant species. Leaf, cotyledons, hypocotyls, cotyledonary meristems, and embryos are among those that have been used successfully.

Because neither the transformation nor the regeneration are 100% effective, the chance of obtaining a transformed plant depends on these two processes occurring consecutively in the same cell. In many cases, the production of transgenic plants is prevented due to the inability to regenerate plants from those tissues susceptible to transformation. For species in which somatic embryogenesis is a viable method of regenerating plants, there are other limitations. Plants regenerated via somatic embryogenesis may show significant somatic variation, altered ploidy, phenotypic abnormalities and poor fertility (Bean, et al. 1997. *Plant Cell Rep* 16:513-519). While regeneration via direct organogenesis overcomes some of these problems, not all plants can be regenerated in this way. Finally, although transformation of many crop plants is possible, it is usually achieved in highly regenerable lines or cultivars, and the elite agriculturally important lines are not usually amenable to transformation. Therefore, introduction of a desired trait into the elite lines has been limited to subsequent traditional breeding methods following transformation of parental lines.

In order to develop a transgenic plant line expressing a new trait, it is desirable to produce a large number of transgenic plants from which the best expressing line can be selected. The requirement for a number of plant lines stems from the fact that the integration of the T-DNA fragment into the plant genome is a random event, and therefore, each transgenic plant will contain the new gene integrated into different sites

of the genome. Due to this phenomenon termed 'position effect', the various transgenic lines will vary in the levels of expression of the introduced gene (Ulian, et al. 1994. "Expression and inheritance pattern of two foreign genes in petunia," *Theor Appl Genet* 88:433-440). Therefore, it is desirable to produce a large number of transgenic lines in order to select for those expressing the introduced gene at a high level.

5 The only plant which has been successfully transformed with a high degree of ease and efficiency is *Arabidopsis thaliana*, a model plant used widely for genetic and molecular analyses of plant developmental processes. A direct method of transformation has been developed for *Arabidopsis thaliana* (Bechtold, et al. 1993. "In 10 planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants," *Comptes Rendus de l'Academie des Sciences Serie III Sciences de la vie* 316.). In this transformation process, the plant is (1) grown to maturity, (2) immersed in a suspension of *Agrobacterium* cells, (3) held under vacuum for a short period of 15 time, and then (4) allowed to set seed. A proportion of the progeny is transformed. Recent data suggest that the gametophyte progenitor, gametophyte, or fertilized embryos are the targets (Bechtold, N. and Pelletier, G. 1998. "In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration," *Methods Mol Biol* 82:259-266). Although Bechtold's method has been tried in other 20 species including *Brassica napus* and *Beta vulgaris*, these attempts reportedly have been unsuccessful (Siemens, J. Scheiler, O. 1996. "Transgenic plants: genetic transformation-recent developments and the state of the art," *Plant Tissue Culture and Biotechnology* 2:66-75).

25 Leguminous crops such as peas, soybean, bean, alfalfa, peanut, chick pea, pigeon pea and clover have widespread economic importance throughout the world. Legumes are an important source of protein as grain and forage legume crops for animals and as grain legumes for humans. For example, soybeans (*Glycine max*) are a major source of protein in animal and human food, and soybean oil is the most widely used edible oil in the world. The productivity, and therefore value, of a wide range of 30 leguminous crops could be increased by the introduction of traits such as disease resistance, herbicide resistance, insect resistance, reduced levels of tannins and lignin

(forage legumes), and improved protein and lipid quality. For example, the soybean cyst nematode causes losses in yield of up to one billion United States dollars per year. With the recent cloning of a beet cyst nematode resistance gene and a potato cyst nematode resistance gene (Williamson, V.M. 1999. *Curr Opin Plant Biol* 2:327-31),
5 strategies are now being explored for genetically engineering resistance in plants.

While there have been some attempts to introduce these traits into leguminous crops via genetic engineering, the current transformation methods involving tissue culture are exceedingly labor intensive and inefficient. In particular, the large seed grain legumes, such as pea, bean, and soybean have proved very difficult to transform,
10 and tissues susceptible to transformation have proven difficult to regenerate.

[Bingham, et al. 1975. "Breeding alfalfa which regenerates from callus tissue in culture," *Crop Science* 15:719-721; Hinchee, et al. 1988. "Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer," *Bio/Technology* 6:915-922; Schroeder, et al. 1993. "Transformation and regeneration of two cultivars of pea
15 (*Pisum sativum* L.)" *Plant Physiol* 101:751-757; Chabaud, et al. 1996. *Plant Cell Rep* 15:305-310; Kar, et al. 1996. *Plant Cell Rep* 16:32-37; Kim and Minamikawa. 1996.
Plant Sci 117:131-138; Trieu and Harrison. 1996. *Plant Cell Rep* 16:6-11; Bean, et al.
1997. *Plant Cell Rep* 16:513-519; Cheng, et al. 1997. *Plant Cell Rep* 16:541-544; and
Dillen, et al. 1997. "Exploiting the presence of regeneration capacity in the Phaseolus
20 gene pool for *Agrobacterium*-mediated gene transfer to the common bean.

Mededelingen-Faculteit-Landbouwkundige-en-Toegepaste-Biologische-Wetenschappen-Universiteit-Gent 62:1397-1402].

In an alternative approach, it was shown that cells within a soybean meristem can be transformed by particle bombardment. However, this leads to chimeric plants with transformed sectors. Some of these sectors will eventually give rise to seed, and the seed will carry the transgene (McCabe, et al. 1988. *Bio/Technology* 6:923-926;
25 Chowrira, et al. 1995. "Electroporation-mediated gene transfer into intact nodal meristems *in planta*: generating transgenic plants without *in vitro* tissue culture," *Molecular Biotechnology* 3:17-23; and Chowrira, et al. 1996. "Transgenic grain legumes obtained by *in planta* electroporation-mediated gene transfer," *Molecular Biotechnology* 5:85-96). While this procedure has enabled the production of transgenic
30

soybean, it is very labor intensive because numerous meristems need to be bombarded to have a realistic chance of obtaining any transgenic seeds.

5 *Medicago truncatula* Gaertn. (barrel medic) is a diploid, autogamous, annual medic that is grown as a pasture legume in a number of regions throughout the world, including Mediterranean areas, South Africa and Australia (Crawford, et al. 1989).

"Breeding annual *Medicago* species for semiarid conditions in Southern Australia," *Adv Agron* 42:399-437). In Australia, the annual medics are the main legume found on over 50 million hectares of agricultural land, and a variety of species and ecotypes have been developed. The first commercial cultivar of *M. truncatula* was sown in 1938, and this 10 species has been favored due to its ability to tolerate both low rainfall and high lime soils (Crawford, et al. 1989. *Adv Agron* 42:399-437). *Medicago truncatula* also is emerging as a model legume for studies of the nitrogen-fixing Rhizobium/legume symbiosis and the arbuscular mycorrhizal symbiosis [Cook, et al. 1995. "Transient induction of a peroxidase gene in *Medicago truncatula* precedes infection by

15 *Rhizobium meliloti*," *Plant Cell* 7:43-55; van Buuren, et al. 1998. "Novel genes induced during an arbuscular mycorrhizal (AM) symbiosis between *M. truncatula* and *G. versiforme*," *MPMI* 12:171-181]. The attributes that make *M. truncatula* a useful 20 model plant for molecular and genetic analyses include its small genome (4.5 times larger than *Arabidopsis*), rapid life cycle, and relatively small physical size (Barker, et al. 1990. "Medicago truncatula, a model plant for studying the molecular genetics of the Rhizobium-legume symbiosis," *Plant Mol Biol Rep* 8:40-49). In addition, it can be transformed via *Agrobacterium* and regenerated via somatic embryogenesis, or alternatively, by direct organogenesis (Thomas, et al. 1992. "Genetic transformation of 25 *Medicago truncatula* using *Agrobacterium* with genetically modified Ri and disarmed Ti plasmids," *Plant Cell Rep* 11:113-117; Chabaud, et al. 1996. *Plant Cell Rep* 15:305-310; Trieu and Harrison. 1996. *Plant Cell Rep* 16:6-11; Hoffmann, et al. 1997. "A new *Medicago truncatula* line with superior in vitro regeneration, transformation, and symbiotic properties isolated through cell culture selection," *Mol Plant-Microbe Interact* 10:307-315).

30 Although *Agrobacterium*-mediated transformation with regeneration via somatic embryogenesis or direct organogenesis is a viable approach, these methods are

very labor intensive, not very efficient, and in some cases, very slow. While these approaches may be suitable for the generation of small numbers of transgenic plants, they cannot be used to generate the large numbers of lines required for many genetic approaches and high through-put systems, such as T-DNA mutagenesis or activation tagging.

An *Agrobacterium*-mediated transformation method has now been found wherein seedlings, rather than flowering plants or tissue explants, are utilized as the subject biological material for exposure to *Agrobacterium* cells. Moreover, following maturation of treated plants and seed set, transgenic plants are selected directly from a population of progeny representing various insertional events. This seedling transformation method provides high efficiency, low labor input, and large numbers of transgenic plants without all the problems associated with transformation of flowering plants or tissue explants and regeneration via somatic embryogenesis or direct organogenesis.

10

15

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts a map of the T-DNA from the binary vector pBI121-*bar*.

Fig. 2(a) is a Southern blot of *Hind*III digested DNA from transgenic plants (progeny of infiltrated plants) hybridized with a *bar* probe. Samples 1.3-1.8 are from Treatment 1 (1-minute vacuum infiltration); Samples 2.2-2.7 are from Treatment 2 (1.15-minute vacuum infiltration); and Samples 3.1-3.5 are from Treatment 3 (40-second vacuum infiltration followed by 20-second hold). The pBI121-*bar* plasmid DNA is included to the left of the blot. This part of the blot was excised and exposed for a shorter time than the rest of the blot to prevent overexposure. C is DNA from a non-transformed control *M. truncatula* plant.

Fig. 2(b) is a Southern blot of *Hind* III digested DNA from transgenic plants (progeny of infiltrated plants) hybridized with a *bar* probe. Samples 1.3-1.8 are from Treatment 1 (1-minute vacuum infiltration); Samples 2.2-2.7 are from Treatment 2 (1.5-minute vacuum infiltration); and Samples 3.1-3.5 are from Treatment 3 (40-second

vacuum infiltration followed by 20-second hold). The pBI121-*bar* plasmid DNA is included to the left. This part of the blot was excised and exposed for a shorter time than the rest of the blot to prevent overexposure. C is DNA from a non-transformed control *M. truncatula* plant.

5 Fig. 3(a) is a Southern blot of *Hind*III digested DNA from transgenic plants (progeny of infiltrated plants) hybridized with a *npt II* probe. Samples 1.6-1.20 are from Treatment 1 (1-minute vacuum infiltration) and Samples 2.12 and 2.13 are from Treatment 2 (1.15-minute vacuum infiltration). C is DNA from a non-transformed control *M. truncatula* plant.

10 Fig. 3(b) is a Southern blot of *Hind*III digested DNA from transgenic plants (progeny of infiltrated plants) hybridized with a *bar* probe. Sample 1.6-1.20 are from Treatment 1 (1-minute vacuum infiltration); and Sample 2.12 and 2.13 are from Treatment 2 (1.5-minute vacuum infiltration). C is DNA from a non-transformed control *M. truncatula* plant.

15 Fig. 4 is an agarose gel showing a portion of the *bar* gene that has been amplified from DNA from transgenic soybean plants via PCR with *bar* specific primers. The arrow points to a 423bp amplified fragment. The lane labeled "M" contains molecular weight markers. The 500 bp marker is indicated. Samples 6-27 are soybean transformants that survived the herbicide treatment. Transformants 6, 13, 14, 20 15, and 16 show an amplified fragment of the correct size.

SUMMARY OF THE INVENTION

In one aspect, the present invention is a method for direct plant transformation using seedlings and *Agrobacterium* comprising: (a) contacting at least one seedling with *Agrobacterium* cells which harbor a vector that enables the *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to the seedling and (b) applying a vacuum to the seedling in contact with the *Agrobacterium* cells at one point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the seedling such that the *Agrobacterium* cells transfer the T-DNA to cells of the seedling at a second point in time, wherein the first point in time

and the second point in time are either the same or different. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct plant transformation using seedlings and *Agrobacterium* comprising: (a) contacting at least one seedling with a mixture of *Agrobacterium* cells, the mixture comprising cells from a *Agrobacterium* strain harboring a vector with a first DNA fragment and cells from the *Agrobacterium* strain harboring the vector with a second DNA fragment, wherein the vector enables the *Agrobacterium* cells to transfer the T-DNA to cells of the seedling; and (b) applying a vacuum to the seedling in contact with the mixture of *Agrobacterium* cells at a first point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the seedling such that the *Agrobacterium* cells transfer at least one gene to cells of the seedling at a second point in time, wherein the first point in time and the second point in time are the same or different. In a preferred method, the vector comprises a selectable marker gene. A preferred selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In another aspect, the present invention is a method for direct plant transformation using seedlings and *Agrobacterium* comprising: (a) contacting at least one seedling with *Agrobacterium* cells which harbor a vector that enables the *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to the seedling; (b) applying a vacuum to the seedling in contact with the mixture of *Agrobacterium* cells at a first point in time, the vacuum being of sufficient strength to force the *Agrobacterium* cells into intimate contact with the seedling such that the *Agrobacterium* cells transfer the T-DNA to cells of the seedling at a second point in time, wherein the first point in time and the second point in time are the same or different; (c) allowing the transformed seedling to grow to maturity and set seed; (d) germinating the seed to form progeny; (e) exposing the progeny to an agent enabling detection of selectable marker gene expression; and (f) selecting for progeny expressing the selectable marker gene and at least one gene, wherein expression of the selectable marker gene and at least one gene indicates gene

transfer. In a preferred method, the selectable marker gene is a herbicide resistance gene. A preferred herbicide resistance gene is a *bar* gene.

In yet another aspect, the present invention is a plant transformed according to the above-described methods of seedling transformation.

5 In yet another aspect, the present invention is a seed from a plant transformed according to the above-described methods of seedling transformation.

In yet another aspect, the present invention is a progeny plant from a seed obtained from a plant transformed according to the above-described methods of seedling transformation.

10

DETAILED DESCRIPTION

A plant transformation process has now been found which utilizes vacuum infiltration of seedlings to introduce *Agrobacterium* T-DNA carrying a selectable marker gene and the gene(s) of interest into the seedlings. A seedling as used herein is defined as a plant from about the beginning of seed germination to about the time true leaves develop. The transformation methods described herein can be applied to the seedlings of any plant, including dicots and monocots which can be successfully transformed by *Agrobacterium*-mediated gene transfer. In particular, leguminous plants are transformed at high rates of efficiency.

20 The transformation method described herein is generally accomplished by growing the *Agrobacterium* strain carrying a gene(s) of interest under selective conditions in liquid culture until it reaches exponential growth phase. The *Agrobacterium* cells are then pelleted by centrifugation and resuspended in a vacuum infiltration medium. The seedlings are immersed in the *Agrobacterium* cell suspension and subjected to vacuum infiltration whereby the *Agrobacterium* cells are then introduced into the seedlings, resulting in infiltrated plants that subsequently produce 25 transformed seed from which a transformed plant is obtained.

The transformation of seedlings is accomplished through *Agrobacterium*-mediated gene transfer. *Agrobacterium* strains useful in the transformation of a

seedling include any aggressive strain which, upon contact with a transformable plant cell, is capable of transferring T-DNA into the cell for integration into the plant's genome. In the transformation method described herein, the *Agrobacterium* strain can carry one plasmid with multiple gene(s) of interest. Alternatively, transformation is performed using a mixture of *Agrobacterium* cells in which the vector carries different fragments of DNA, e.g., selected fragments from a specific DNA library. To achieve the optimum transformation rate in a given plant, the *Agrobacterium* strain which provides the greatest number of transformed seedlings is selected. For leguminous plants, *Agrobacterium tumefaciens* EHA105, ASE1, and Gv3101 strains are preferably utilized. The gene(s) of interest can be transformed into the *Agrobacterium* by any means known in the art. For example, a DNA fragment modified to contain the gene(s) of interest can be inserted into the T-DNA of an *Agrobacterium* Ti plasmid which also contains genes required to generate the transformed state.

Other modifications of the *Agrobacterium* plasmid T-DNA can be made to assist in the transformation process. For example, to distinguish seedlings which are successfully transformed, a selectable marker gene can be incorporated into the T-DNA of *Agrobacterium* plasmid. Selectable marker genes useful in the transformation methods described herein include any selectable marker gene which can be incorporated into the *Agrobacterium* T-DNA and upon expression, can distinguish transformed from non-transformed progeny. Exemplary selectable markers include a neomycin transferase gene or phosphinothricin acetyl transferase (*bar*) gene. For example, a preferable selection marker is the *bar* gene encoding phosphinothricin acetyl transferase which confers resistance to phosphinothricin-based herbicides. Preferably, the selection marker gene and gene(s) of interest are incorporated into any vector suitable for use with transforming *Agrobacterium* strains. For example, the binary vector, pBI121 vector (Clontech, Palo Alto, CA) can be modified wherein a copy of a phosphinothricin acetyl transferase (*bar*) gene is inserted, under the control of a 35S promoter and octopine synthase 3' sequences, into the *Hind*III site of the T-DNA. The *bar* gene encodes phosphinothricin acetyl transferase which confers resistance to phosphinothricin-based herbicides, such as Ignite® (AgroEvo, Frankfurt, Germany). This selectable marker enables easy selection of transformed plants: upon spraying the

plants with phosphinothricin (PPT) containing herbicides, only transformed plants containing the *bar* gene survive exposure to the herbicide.

In the seedling transformation process, the starter seeds can be pretreated to optimize their germination and to prepare the resulting seedlings for transformation. Surface-sterilization of the seeds is preferred to remove any interfering microorganisms which might infect the germinating seed. Any sterilization means which does not deleteriously affect the seeds can be used. Exemplary methods include use of aqueous 20-30% sodium hypochlorite or 70% ethanol. Preferably, the seeds are sterilized in a solution of 30% sodium hypochlorite and 0.1% Tween 20 for approximately 5 minutes and then thoroughly rinsed to remove the sterilizing solution. Preferably, sterile double distilled or deionized water, or water with reduced oxidizable carbon following reverse osmosis, ion exchange and/or activated charcoal treatment is used to rinse the seeds. Some seeds, for example *M. truncatula*, experience a prolonged dormancy period resulting in delayed germination. These seeds can be treated by a scarification process capable of breaking the dormancy. For example, cracking or scratching the seed coat, soaking the seed to soften the seed coat, or a controlled acid treatment can be utilized. Preferably, a treatment in concentrated sulfuric acid for approximately 10 minutes followed by thorough rinsing to remove the acid is utilized. Preferably, sterile double distilled or deionized water, or water with reduced oxidizable carbon following reverse osmosis, ion exchange and/or activated charcoal treatment is used to rinse the seeds.

After pretreatment, the seeds are placed on a medium capable of supporting germination and subsequent growth of the seedlings. For example, the seeds can be placed on the surface of sterile filter paper or paper towels. Preferably, the seeds are spread on the surface of firm, sterile water agar in petri plates. The seeds are then placed under environmental conditions capable of inducing germination and supporting development of seedlings. Vernalization may be preferred for certain plants such as *M. truncatula* to promote early flowering. Incubation of the resulting seedlings is continued until the seedlings reach an appropriate stage of development for vacuum infiltration. The optimum age of seedlings for vacuum infiltration varies for different plants. In general, seedlings in which the radical has emerged and grown to at least about 1 cm are sufficiently mature. However, since plants develop at different rates,

vacuum infiltration can be optimized for a specific plant by screening seedlings at various stages of development using the methods disclosed herein and determining the stage at which the transformation efficiency is maximized. The time and temperature of incubation can also be adjusted to provide optimum conditions for a specific variety.

5 For example, approximately 15 days after the seeds are placed on germination medium, *M. truncatula* and soybean seedlings are sufficiently matured for vacuum infiltration.

A few days prior to vacuum infiltration, the transforming *Agrobacterium* is subcultured on a general plated growth medium preferably containing appropriate antibiotics to distinguish transformed *Agrobacterium* cells. For example,

10 *Agrobacterium tumefaciens* EHA105 and Gv1301 carrying the *bar* gene are preferably cultured on YEP medium as defined in Example 1 containing rifampicin (20mg/l) and kanamycin (50 mg/l). The *Agrobacterium* cultures are grown at about 28°C for about 2-3 days.

One day prior to the vacuum infiltration, a liquid *Agrobacterium* culture is prepared by aseptically transferring an appropriate inoculum into a general growth medium suitable for growing *Agrobacterium*. TY liquid medium and YEP liquid medium containing appropriate antibiotics to select for the transformed *Agrobacterium* are preferred for *Agrobacterium* EHA105 and Gv1301. The liquid cultures are grown under conditions which provide the *Agrobacterium* to reach exponential growth.

20 Preferably, the liquid culture is incubated at about 28°C in a shaker incubator at about 250 rpm overnight. It is essential to use fresh *Agrobacterium* to achieve transformation.

To provide optimal conditions for transformation, the vacuum infiltration is preferably performed using the transforming *Agrobacterium* liquid culture in exponential growth phase ($OD_{600} = 1.6$). The *Agrobacterium* cells in the liquid culture are pelleted by centrifugation and resuspended in two volumes of a vacuum infiltration medium (e.g., *Agrobacterium* cells grown in 15ml liquid culture, pelleted by centrifugation, and then resuspended in 30ml vacuum infiltration medium). Any plant growth medium capable of supporting the infiltration process and the *Agrobacterium* within the plant while being compatible with plant growth can be used as the vacuum

infiltration medium. More preferably, the vacuum infiltration medium comprises acetosyringone which induces the vir genes of the *Agrobacterium*. For leguminous plants, the vacuum infiltration medium defined in Examples 1 and 2 is preferably utilized.

5 To perform the vacuum infiltration, the seedlings are removed from the germination/incubation medium and placed in any clean container capable of holding several seedlings as well as a volume of vacuum infiltration medium to partially cover the seedlings. Petri plates are useful for this purpose, using about 30-40 seedlings per plate. The *Agrobacterium* suspension in the vacuum infiltration medium is added to the 10 container to wet and partially cover the seedlings. For a standard petri plate, approximately 10ml of the suspension is sufficient. The petri plate containing the seedlings in *Agrobacterium* suspension is placed in a vacuum chamber. The preferred amount of vacuum to be used in the transformation process is the minimal amount necessary to force the *Agrobacterium* into the apoplastic spaces of the seedlings.

15 Approximately 28mmHg was sufficient for transforming *M. truncatula* and soybean. The time and manner in which the vacuum is applied to the seedlings depends upon the plant and has to be determined empirically. The vacuum can be applied then released. Alternately, the vacuum can be applied, released, reapplied, and then released again. The duration of vacuum can vary from about 0.1 to about 5 min, more preferably from 20 about 0.5 to about 2 min, and most preferably for about 1 min. For *M. truncatula*, plants held under vacuum for 0.5 min and for 2 min gave rise to transgenic plants, but plants held under vacuum for 1 min gave the maximum transformation efficiency.

25 Following vacuum infiltration, the *Agrobacterium* suspension is decanted, and the seedlings are blotted on sterile filter paper or blotting paper. The seedlings can then be planted into a complete soil mix that will allow full growth and development of the plant and the production of seed. The seedlings are then permitted to mature and set seed. Preferably, the plants are kept at a humidity, temperature, duration of photo period, and spectrum of light which favor plant growth. To increase the viability of the transformed plants and to improve the transformation efficiency, the seedlings are 30 optionally incubated on a co-cultivation medium for 2-3 days prior to planting in a complete soil mix. Any co-cultivation medium which supports growth of the seedlings

can be used. For leguminous plants, the co-cultivation medium given in Examples 1 and 2 is preferred. The plants are then permitted to develop to maturity and set seed. A portion of the seeds will carry the transgene in their genomes. The seeds are germinated, and the resulting progeny which exhibit stable inheritance of the transgene are selected.

Several methods known in the art can be used to distinguish the progeny exhibiting stable inheritance of the transgene. For transgenic plants wherein the gene(s) of interest results in a visible phenotypic change, the selection can be based upon visual examination of the progeny. For plant transformations involving *Agrobacterium* carrying plasmids containing a selectable marker gene, the appropriate selectable agent can be applied to the plants to select the transformants. Optionally, Southern blot analysis or PCR analysis can be used to verify the presence of the transferred gene in the genome of the transformed plants.

The seedling transformation processes of the present invention are further illustrated in detail in the examples provided below. While these examples describe the invention, it is understood that modifications to the methods to optimize transformation of a specific plant are well within the skill of one in the art, and such modifications are considered within the scope of the invention.

Example 1: Transformation of *M. truncatula* by Vacuum-infiltration of Seedlings

M. truncatula seedlings were transformed to incorporate the *bar* gene and the *nptII* gene into the plant's genome using the transformation process of the present invention.

Preliminary

Prior to transformation, a modified version of the binary vector, pBI121 vector (Clontech, Palo Alto, CA) was made by inserting a copy of a phosphinothricin acetyl transferase (*bar*) gene, under the control of a 35S promoter and octopine synthase 3' sequences, into the *Hind*III site of the T-DNA to create a plasmid called pBI121-*bar* (Fig. 1). The construct was confirmed by restriction analysis and PCR analysis, and then transformed into an *Agrobacterium tumefaciens* strain EHA105 (Hood, et al. 1993. "New *Agrobacterium* helper plasmids for gene transfer to plants," *Trans Res* 2:208-

218). Additional constructs in *Agrobacterium* were also obtained as given in Table III.

While the following procedure is presented for the pBI121-*bar* in the EHA105 *Agrobacterium* strain, the same procedure was followed for the other constructs with the exception that the growth medium was supplemented with specific antibiotics necessary for maintaining the plasmid.

5

Day 1:

The *M. truncatula* seed was sterilized and germinated as follows. The seeds were soaked in conc. H₂SO₄ for approximately 10 min. The acid was removed, and the seeds were rinsed extensively in sterile cold double distilled water. This treatment was used to break dormancy in *M. truncatula*.

10 The seeds were then surface-sterilized by soaking the seeds in a sterilizing solution such as 30% Clorox / 0.1% Tween 20 solution for approximately 5 min with gentle agitation. The seeds were rinsed extensively with sterile cold double distilled water.

15 The seeds were then spread on a firm water-agar (for example, 0.8%) (Sigma Chemical Co., St. Louis, MO) in petri plates. The water-agar petri plates containing the seeds were wrapped with aluminum foil and kept at 4°C for 15 days. This vernalization step was used to promote early flowering of *M. truncatula*.

About Day 12:

20 *Agrobacterium tumefaciens* EHA105 carrying pBI121-*bar* was subcultured for isolation onto a fresh agar plate containing YEP medium [1 liter: 10g Bacto-peptone (Difco, Detroit, MI); 10g yeast extract; 5g NaCl; and 15g Bacto-agar (Difco, Detroit, MI) at pH=6.8 without adjusting] containing rifampicin (20 mg/l) and kanamycin (50 mg/l), and the subculture was incubated at approximately 28°C for about 2-3days.

25 **About Day 14:**

One loop, or approximately 3 large colonies of the *Agrobacterium* subculture was inoculated into about 15 ml TY liquid medium [1 liter: 5g tryptone, 3g yeast extract, 0.88g CaCl₂·2H₂O at pH=7] containing rifampicin (20 mg/l) and kanamycin (50 mg/l) and incubated on a 28°C shaker at 250 rpm overnight.

About Day 15:

The *Agrobacterium* liquid culture was grown until an exponential phase (OD₆₀₀ 1.6) was reached. The *Agrobacterium* cells were pelleted by centrifugation and resuspended in 30 ml of vacuum-infiltration medium (VIM) [1 liter: 10ml PDM salt solution at 100X concentration (400ml at 100X: 100g KNO₃; 12g NH₄H₂PO₄; 16g MgSO₄·7H₂O; 0.4g MnSO₄·H₂O; 0.2g H₃BO₃; 0.008g CuSO₄·5H₂O; 0.04g KI; 0.004g CoCl₂·6H₂O; 0.04g ZnSO₄·7H₂O, and 0.004g Na₂MoO₄·2H₂O; filter sterilized and stored at room temperature); 10ml PDM iron and vitamins (1 liter at 100X: 0.5g nicotinic acid; 0.05g pyridoxine·HCl; 0.5g thiamine·HCl; 100g myo-inositol; 1.5g FeSO₄·7H₂O; and 2g Na₂EDTA; filter sterilized; stored for immediate use at 4°C; long term storage at -20°C); 0.2g CaCl₂·H₂O; 1.5ml of 10mM benzylaminopurine (BAP; 0.0565g in 0.15ml 2N NaOH and 24.85ml double distilled H₂O stored at 4°C); 0.05ml of 10mM alpha-naphthaleneacetic acid (NAA; 0.0465g in 3ml 95% ethanol and 22ml 70% ethanol stored at 4°C); 10g sucrose; and 0.1ml acetosyringone (AS; 1M in DMSO stored at -20°C), wherein PDM salts, PDM iron and vitamins, CaCl₂·H₂O and sucrose are combined, the pH adjusted to 5.8 with KOH and autoclaved on liquid cycle for 20 min, and when the medium cools to 50°C, BAP, NAA, and AS are added].

The seedlings were removed from the water agar plates and placed in a clean standard petri dish at approximately at 30-40 *M. truncatula* seedling per petri plate. Approximately 10ml of the *Agrobacterium* suspension in the vacuum infiltration medium was added to the petri plate, a volume sufficient to wet and partially cover the seedlings. The petri plates containing the seedlings wetted with *Agrobacterium* suspension were placed in a vacuum chamber. Three methods of vacuum infiltration were tested. In Treatment 1, a vacuum was drawn to 28mmHg for approximately 1 min, released rapidly, redrawn to 28mmHg for approximately 1 min, and finally released rapidly. In Treatment 2, a vacuum was drawn to 28mmHg for approximately 1.5 min, released rapidly, redrawn to 28mmHg for approximately 1.5 min, and finally released rapidly. In Treatment 3, a vacuum was drawn to 28mmHg for approximately 40 seconds, held for 20 seconds, and finally released rapidly. For all treatments, the seedlings were then blotted on sterile filter paper or blotting paper and spread onto petri plates containing co-cultivation medium(CM) [1 liter: 10ml PDM salt solution at 10X

concentration; 10ml PDM iron and vitamins; 0.2g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$; 10g sucrose; 7.5g agar-agar (Sigma Chemical Co., St. Louis, MO); and 0.1ml AS, wherein PDM salts, PDM iron and vitamins, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, agar-agar, and sucrose are combined, the pH adjusted to 5.8 with KOH and autoclaved on liquid cycle for 20 min, and when the medium cools to 50°C, AS is added]. The seedlings were incubated in a growth chamber under the conditions given in Table I for approximately 2-3 days.

Table I: Growth Chamber Conditions

	Temp. (°C)	Humidity (%)	Photo-period	Light
SET	20 (Day & Night)	90	16 hrs (8am-12midnight)	Top light only (1/2 fluorescent, no incandescent) 4 light bulbs Sylvania 115W, F48T1

About Day 17:

The seedlings were washed twice with H_2O and then planted in pots in Metro-mix 200 soil mixture. To allow the plants to adjust slowly to ambient humidity the following procedure was followed: the pots containing the seedlings were initially covered with a plastic cover; after one week, the cover was propped open, and after a couple of days, the cover was removed completely. The plants were allowed to mature under conditions that are suitable for optimal plant growth, i.e., at 22-25°C with eighteen hour days.

About Day 40

The plants began to flower at approximately 24 days after planting in soil. The resulting seeds were collected and germinated under conditions optimal for germination, i.e., a short cold treatment for four days on a damp filter paper, left at room temperature for 1-2 days, and then planted in soil. When the seedlings had a few leaves (approximately 15 days old), they were sprayed with 80 mg/L PPT (~1/7000 dilution of 600mg/ml solution stored at -20°C), and the results are presented below.

In this study, 120 *M. truncatula* seedlings were vacuum infiltrated with *Agrobacterium tumefaciens* strain EHA105 carrying the pBI121-*bar* plasmid as described above. Three different treatments were used, and forty seedlings were infiltrated per treatment. The treatments varied only in the length of time of the vacuum infiltration treatment. The infiltrated plants were permitted to mature and set seed. The progeny seedlings were sprayed with Ignite (PPT) and resistant seedlings were further analyzed for the presence of the *bar*, *nptII* and B-Glucuronidase (GUS) genes. The results are shown in Table II . Fig. 2A, 2B, 3A, and 3B present data obtained in Experiments T-84-1, T84-2, and T84-3 of Table II. These results show the efficiency of transformation ranging from 2.9% to 27.6% for the various transformation experiments.

Table II: Transformants Resulting from Transformation of *M. truncatula*
via Infiltration of Seedlings with *Agrobacterium*

Experiment ^a	Construct and <i>Agrobacterium</i> ^b	No. of Plants Infiltrated	Approx. No. of Seed Collected	No. of Seedlings Germinated	No. of Seedlings resistant to PPT	% transformation	% independent transformants (*)
T84-1	pBI121- <i>bar</i> /EHA105	40	1326	329	22	6.7	78
T84-2	pBI121- <i>bar</i> /EHA105	40	1263	302	16	5.3	ND ^c
T84-3	pBI121- <i>bar</i> /EHA105	40	1214	173	5	2.9	ND
T87-1	pGA482- <i>bar</i> /EHA105	40	ND	217	10	4.6	86
T87-2	pGA482- <i>bar</i> /EHA105	40	ND	502	89	17.7	78
T87-3	pKYLX71Gus /EHA105	40	ND	ND	**	ND	ND
T87-4	pBINmgfp-ER- <i>bar</i> / EHA105	40	ND	382	13	3.4	8
T87-7	pBINmgfp-ER- <i>bar</i> and pKYLX71Gus / EHA105	40	ND	210	58	27.6	ND
T88	pSKI015/ Gv3101	70	ND	565	40	7.1	ND

^a The following treatment methods were used: T84-1, T87-1, T87-2, T87-3, T87-4, T87-7, and T88, infiltration for 1 min (2X); T84-2, infiltration for 1.5 min (2X); T84-3, infiltration for 40 sec and hold 20 sec.

^b The following *Agrobacterium tumefaciens* strains and binary vectors were used in these experiments: *A. tumefaciens* strain ASE1 carrying the binary vectors pSLJ525 (Jones et al. 1992. "Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants," *Trans. Res.* 1: 285-297) or pSKI006 (<http://www.salk.edu/LABS/pbio-w/>). *A. tumefaciens* strain EHA105, carrying pBI121-bar or PKYLX7-Gus (Franklin et al. 1993 "Genetic transformation of green bean callus via *Agrobacterium* mediated DNA transfer," *Plant Cell Rep* 12:74-79), or pBINmgfp-ER-bar, or pGA482-bar. *A. tumefaciens* strain Gv3101 carrying pSKI015 (Kardailsky et al. 1999. "A pair of related genes with antagonistic roles in floral induction," *Science* 286, 1962-1965). The addition of the bar gene to a number of vectors was achieved as follows. A *Hind*III/*Hpa*I fragment containing the 35S-bar-OCS 3' sequences cassette was excised from pSLJ525 and inserted between the *Hind*III and *Hpa*I sites of pGA482 to create pGA482-bar. The same *Hind*III/*Hpa*I fragment was used to produce pBI121-bar and pBINmgfp-ER-bar. For each of these vectors, the *Hpa*I site was converted to a *Hind*III site by the addition of an *Hpa*I-*Hind*III linker and then the *Hind*III fragment was then inserted into the *Hind*III site of pBI121 or pBINmgfp-ER (Haseloff et al. 1997 "Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly," *Proc. Natl. Acad. Sci. USA* 94: 2122-2127(1997) to create pBI121-bar and pBINmgfp-ER-bar respectively.

^c ND=not determined.

Transformed plants from Treatments T84-1, T84-2, and T84-3 were also analyzed by Southern blot analysis to demonstrate the presence of the transgene within the plant. DNA was isolated from 22 transgenic plants (12 plants from Treatment T84-1, 6 plants from Treatment T84-2, and 4 plants from Treatment T84-3) and digested with restriction enzyme *Hind*III. The digested DNA was separated by electrophoresis and blotted to nylon membranes. The membranes were probed with an internal DNA fragment (450bp) of the bar gene labeled with ³²P-dATP. All of the transformed plants

contained DNA fragments that hybridized to the *bar* probe indicating that this gene is integrated into the genome (Fig. 2A and 2B). The plasmid pBI121-*bar* DNA was included on the blot as a positive control, and a sample of DNA from non-transformed *M. truncatula* plant was included as a negative control. As expected the *bar* probe hybridized to the plasmid DNA but not to the DNA from the non-transformed *M.* 5 *truncatula* plant (Fig. 2A and 2B). The expected size of the hybridizing fragment from the plasmid is 1.6kb; however, all of the fragments were larger, probably due to a rearrangement towards the left border. The blots were stripped and reprobed with an internal fragment of the *nptII* gene (766bp) labeled with ^{32}P -dATP. The *nptII* gene is 10 carried on the pBI121-*bar* plasmid, between the right border of the T-DNA and the *bar* gene (Fig. 1). All of the transformed plants contained DNA fragments that hybridized to the *nptII* probe indicating that this gene is also integrated into the genome. This combination of digest and probe provided a right border analysis and demonstrated the presence of independent transformants. For example, the unique *bar*-hybridizing 15 fragments shown with Transformants 1.6, 1.10, 1.11, 1.14, 1.13, and 2.12 provided evidence that these are independent transformants. Again, the non-transformed control plant does not contain DNA capable of hybridizing to this probe (Fig. 3A and 3B). The pBI121-*bar* T-DNA also contains a copy of the GUS gene between the *bar* gene and the left border (Fig. 1); however, this gene could not be detected in the transformed 20 plants. Loss of genes located between the selectable marker and the left border have been previously reported; thus, the lack of the GUS gene in the transformed plants confirmed these findings. These results were consistent with the *bar* Southern analysis and offered an explanation of the larger than expected *bar* hybridizing fragment. Thus, it was demonstrated that the GUS gene or any gene of interest must be inserted in the 25 plasmid between the *bar* gene and the right border (at the location of the *nptII* gene) to ensure integration.

Seed from the transgenic plants were collected and germinated, and the resulting seedlings were sprayed with PPT herbicide. The progeny of the transgenic plants were also highly resistant to PPT, indicating that the transgenes are stable and inherited by the following generation. As shown in Table III, data was obtained which 30

showed that the transgenes were inherited in a stable Mendelian fashion. The results show that the lines can be propagated past the T1 generation.

Table III: Segregation Analysis (Phosphinothricin Resistance) of Progeny from a Selection of Transformants Prepared by Infiltration of Seedlings

Progeny from transformants	Number of plants resistant to phosphinothricin (R)	Number of plants sensitive to phosphinothricin (S)	Chi-square test against ratios (p) ^a		
			3R:1S	15R:1S	63R:1S
T84-1.14	83	0	-	*	**
T84-1.19	44	20	**	-	-
T84-1.20	38	14	**	-	-
T84-2.6	44	21	**	-	-

^a ** = p value of ≥ 0.05 ; * = p value of ≥ 0.01 ; - = p value of ≤ 0.01 .

Example 2: Transformation of Soybean by Vacuum-infiltration of Seedlings

Soybean seedlings were transformed to incorporate the *bar* gene into the plant's genome using the transformation process of the present invention.

Day 1:

The seeds were then surface-sterilized by soaking the seeds in 20% sodium hypochlorite for approximately 5 min with gentle agitation. The seeds were rinsed for eight times in sterile double distilled water. The seeds were then placed in a large volume of water and allowed to imbibe at room temperature for 3-12 hours.

The seeds were then spread on a firm water-agar (for example, 0.8%) (Sigma Chemical Co., St. Louis, MO) in petri plates. The water-agar petri plates containing the seeds were wrapped with aluminum foil and kept at 18-20°C for 15 days.

About Day 12:

Agrobacterium tumefaciens Gv3101 which carries the SKI015 vector with a copy of the *bar* gene was subcultured for isolation onto a fresh agar plate containing YEP medium [1 liter: 10g Bacto-peptone (Difco); 10g yeast extract; 5g NaCl; and 15g Bacto-agar (Difco) at pH=6.8 without adjusting] containing rifampicin (10 mg/l),

kanamycin (50 mg/l), carbenicillin (50 mg/l), and gentamicin (20 mg/l), and the subculture was incubated at approximately 28°C for about 2-3 days.

About Day 14:

One loop, or approximately 3 large colonies of the *Agrobacterium* subculture was inoculated into about 15 ml TY liquid medium [1 liter: 5g tryptone, 3g yeast extract, 0.88g CaCl₂·2H₂O at pH=7] containing rifampicin (10 mg/l), kanamycin (50 mg/l), carbenicillin (50 mg/l), and gentamicin (20 mg/l) and incubated on a 28°C shaker at 250 rpm overnight.

About Day 15:

The *Agrobacterium* liquid culture was grown until an exponential phase (OD₆₀₀ 1.6) was reached. The *Agrobacterium* cells were pelleted by centrifugation and resuspended in 30 ml of vacuum-infiltration medium (VIM) [1 liter: 10ml PDM salt solution at 10X concentration; 10ml PDM iron and vitamins; 0.2g CaCl₂·H₂O; 1.5ml of 10mM benzylaminopurine (BAP; 0.0565g in 0.15ml 2N NaOH and 24.85ml double distilled H₂O stored at 4°C); 0.05ml of 10mM alpha-naphthaleneacetic acid (NAA; 0.0465g in 3ml 95% ethanol and 22ml 70% ethanol stored at 4°C); 10g sucrose; and 0.1ml acetosyringone (AS; 1M in DMSO stored at -20°C), wherein PDM salts, PDM iron and vitamins, CaCl₂·H₂O and sucrose are combined, the pH adjusted to 5.8 with KOH and autoclaved on liquid cycle for 20 min, and when the medium cools to 50°C, BAP, NAA, and AS are added].

The seedlings were removed from the water agar plates and placed in a clean standard petri dish at approximately at 10-20 soybean seedlings per petri plate. Approximately 20ml of the *Agrobacterium* suspension in the vacuum infiltration medium was added to the petri plate, a volume sufficient to wet and partially cover the seedlings. The petri plates containing the seedlings wetted with *Agrobacterium* suspension were placed in a vacuum chamber. A vacuum was drawn to 28mmHg for approximately 2 min, released rapidly, redrawn to 28mmHg for approximately 2 min, and finally released rapidly. The seedlings were then blotted on sterile filter paper or blotting paper and spread onto petri plates containing co-cultivation medium(CM) [1 liter: 10ml PDM salt solution at 10X concentration; 10ml PDM iron and vitamins; 0.2g

CaCl₂·H₂O; 10g sucrose; 7.5g agar-agar (Sigma Chemical Co., St. Louis, MO); and 0.1ml AS, wherein PDM salts, PDM iron and vitamins, CaCl₂·H₂O, agar-agar, and sucrose are combined, the pH adjusted to 5.8 with KOH and autoclaved on liquid cycle for 20 min, and when the medium cools to 50°C, AS is added]. The seedlings were 5 incubated in a growth chamber under the conditions given in Table IV for approximately 6-7 days until the *Agrobacterium* could be seen growing around the seedlings on the media.

Table IV: Growth Chamber Conditions

	Temp. (°C)	Humidity (%)	Photo-period	Light
SET	20 (Day & Night)	90	16 hrs (8am-12midnight)	Top light only (1/2 fluorescent, no incandescent) 4 light bulbs Sylvania 115W, F48T1

10 **About Day 17:**

The seedlings were washed twice with H₂O and then planted in pots in Metro-mix 200 soil mixture. To allow the plants to adjust slowly to ambient humidity the following procedure was followed: the pots containing the seedlings were initially covered with a plastic cover; after one week, the cover was propped open, and after a 15 couple of days, the cover was removed completely. The plants were allowed to mature under conditions for optimal growth in a greenhouse.

About Day 40

The plants began to flower, and the resulting seeds were collected and germinated by soaking in water for three hours followed by immediate planting in soil. 20 When the seedlings had one leaf, they were sprayed with 100 ml/l PPT (~1/6000 dilution of 600mg/ml solution in 0.1% Tween 20 stored at -20°C), and the results are presented below.

In this study, 30 soybean seedlings were vacuum infiltrated with *Agrobacterium tumefaciens* strain Gv3101 carrying the SKI015-bar plasmid as described above. 25 Fourteen of the thirty infiltrated soybean seedlings survived, were transplanted into

pots, and allowed to mature and set seed. Approximately 700 progeny seeds were collected, germinated and grown in vermiculite. After the seedlings developed at least one true leaf, they were sprayed with PPT herbicide. PCR analysis was performed to confirm that the plants which survived the PPT herbicide carried the *bar* gene in their genomes. DNA was extracted from each surviving plant and used as a template in a PCR reaction with *bar* specific primers. A fragment of the expected size was amplified from the DNA samples from at least eleven of the transformed plants, indicating that these plants had been transformed. The frequency of transformation was around 1.57%.

In summary, the seedling transformation process described herein is more efficient and less labor intensive than previously reported methods. In addition, somatic alterations are avoided, and direct introduction of genetic material into elite lines is made possible. Large numbers of transgenic plants representing diverse integration events can be generated very rapidly and efficiently, and the transgenes are stable and inherited by the subsequent generation. The major difficulty with regeneration of *Agrobacterium* transformed cells through tissue culture is avoided in the transformation procedures of the present invention, making it useful for legumes such as, soybean, bean and peas for which subsequent regeneration of *Agrobacterium* transformed cells is problematic.

WE CLAIM:

1. A method for direct plant transformation using seedlings and *Agrobacterium* comprising:

(a) contacting at least one seedling with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to said seedling;

(b) applying a vacuum to said seedling in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said seedling such that said *Agrobacterium* cells transfer said T-DNA to cells of said seedling at a second time, wherein said first and second time are the same or different.

2. The method of Claim 1, further comprising:

(c) allowing said seedling to grow to maturity and set seed;

(d) germinating said seed to form progeny; and

(e) selecting for progeny expressing said transferred gene.

3. The method of Claim 1, wherein said vector comprises a selectable marker gene.

4. The method of Claim 2, wherein said vector comprises a selectable marker gene.

5. The method of Claim 3, wherein said selectable marker gene comprises a herbicide resistance gene.

6. The method of Claim 4, wherein said selectable marker gene comprises a herbicide resistance gene.

7. The method of Claim 5, wherein said herbicide resistance gene comprises a *bar* gene.

8. The method of Claim 6, wherein said herbicide resistance gene comprises a *bar* gene.

9. A method for direct plant transformation using seedlings and *Agrobacterium* comprising:

(a) contacting at least one seedling with a mixture of *Agrobacterium* cells, said mixture comprising cells from a *Agrobacterium* strain harboring a vector with a DNA fragment and cells from said *Agrobacterium* strain harboring said vector a second DNA fragment, said vector enabling said *Agrobacterium* cells to transfer said T-DNA to said seedling;

(b) applying a vacuum to said seedling in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said seedling such that said *Agrobacterium* cells transfer T-DNA to cells of said seedling at a second time, wherein said first and second time are the same or different.

10. The method of Claim 9, further comprising:

- (c) allowing said seedling to grow to maturity and set seed;
- (d) germinating said seed to form progeny; and
- (e) selecting for progeny expressing said transferred gene.

11. The method of Claim 9, wherein said vector comprises a selectable marker gene.

12. The method of Claim 10, wherein said vector comprises a selectable marker gene.

13. The method of Claim 11, wherein said selectable marker gene comprises a herbicide resistance gene.

14. The method of Claim 12, wherein said selectable marker gene comprises a herbicide resistance gene.

15. The method of Claim 13, wherein said herbicide resistance gene comprises a *bar* gene.

16. The method of Claim 14, wherein said herbicide resistance gene comprises a *bar* gene.

18. A method for direct plant transformation using seedlings and *Agrobacterium* comprising:

(a) contacting at least one seedling with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to said seedling;

5 (b) applying a vacuum to said seedling in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said seedling such that said *Agrobacterium* cells transfer said T-DNA to cells of said seedling at a second time, wherein said first and second time are 10 the same or different;

15 (c) allowing said transformed seedling to grow to maturity and set seed;

(d) germinating said seed to form progeny;

(e) exposing said progeny to an agent enabling detection of selectable marker gene expression;

15 (f) selecting for progeny expressing said selectable marker gene and at least one gene, said expression of said selectable marker gene and at least one gene indicating gene transfer.

19. The method of Claim 18, wherein said selectable marker gene comprises a herbicide resistance gene.

20. The method of Claim 19, wherein said herbicide resistance gene comprises a *bar* gene.

21. A plant transformed according to the method of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20.

22. Seed from a plant transformed according to the method of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20.

23. A progeny plant from a seed obtained from a plant transformed according to the method of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20.

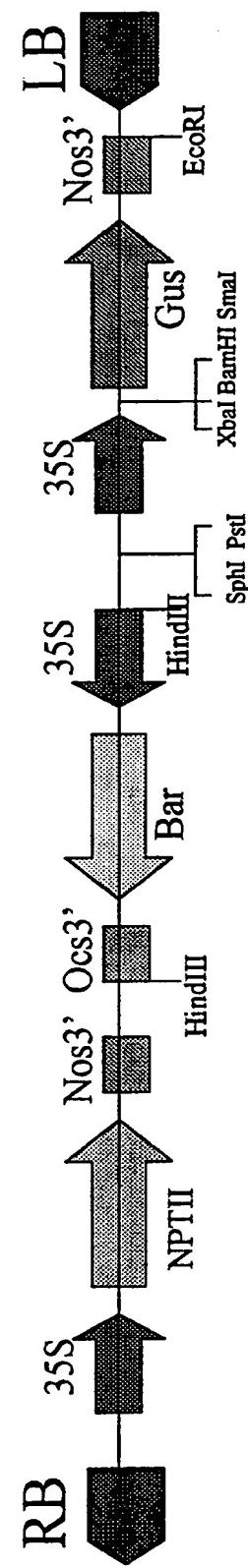


Fig. 1

pBI121-*bar*

C 1.3 1.4 1.5 1.8 2.2 2.4 2.6 2.7 3.1 3.3 3.4 3.5

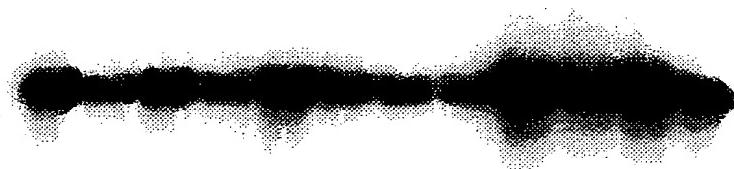


Fig.2a

3/6

pBI121-*bar*

C 1.3 1.4 1.5 1.8 2.2 2.4 2.6 2.7 3.1 3.3 3.4 3.5

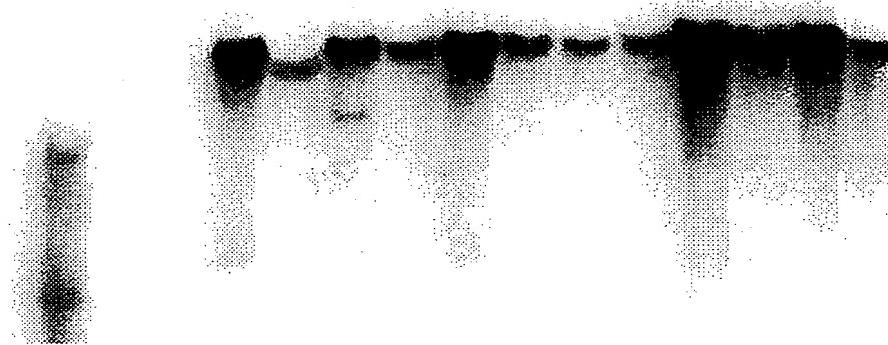
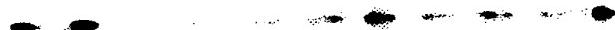


Fig.2b

4/6

1.6 1.10 1.11 1.13 1.14 1.16 1.19 1.20 2.12 2.13 C

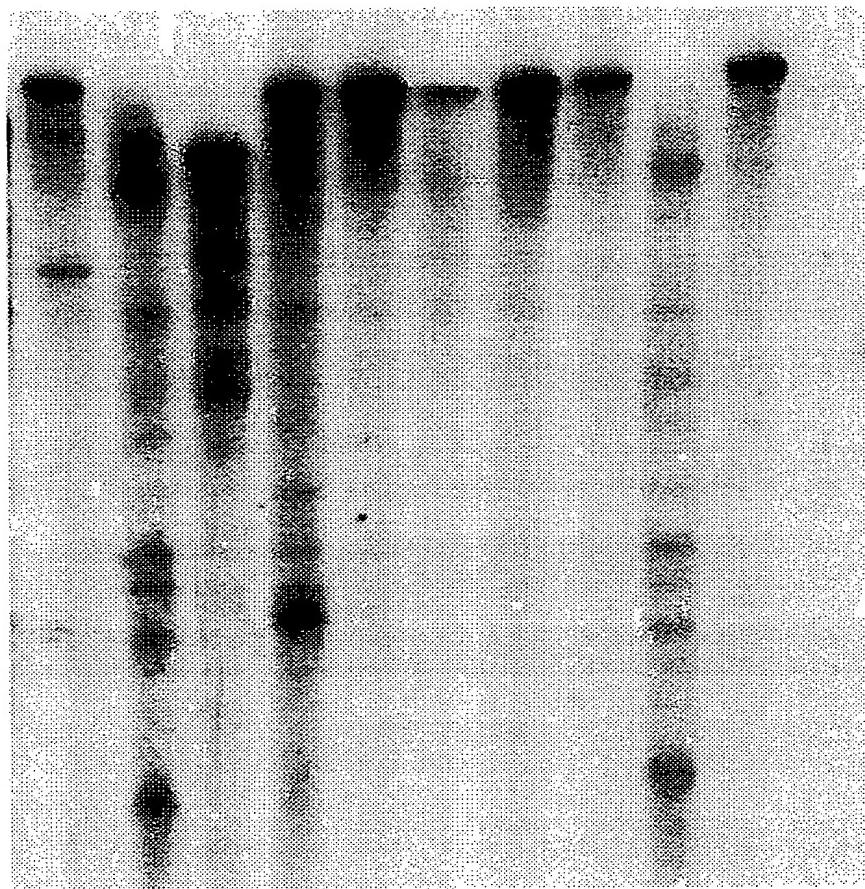


Fig.3a

5/6

1.6 1.10 1.11 1.13 1.14 1.16 1.19 1.20 2.12 2.13 C

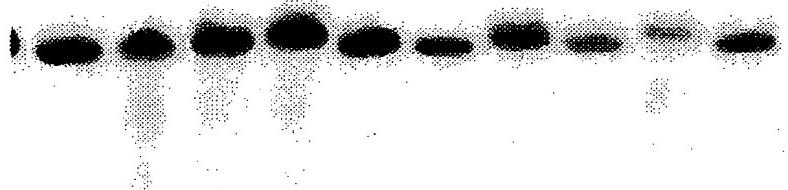


Fig.3b

WO 00/37663

PCT/US99/30972

6/6

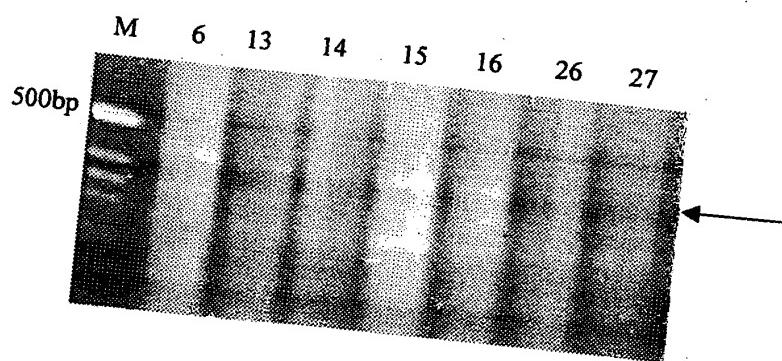


Fig. 4